AMRL-TR-79-89

DEVELOPMENT OF ANALYTICAL TECHNIQUES FOR TOXICOKINETIC RESEARCH

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TECHNICAL REVIEW AND APPROVAL

AMRL-TR-79-89

The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals, "Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

ANTHONY A. THOMAS, MD
Director
Toxic Hazards Division
Air Force Aerospace Medical Research Laboratory

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This report describes the methods developed for the analysis of monomethylhydrazine (MMH) and its metabolites in the urine and blood of laboratory rats which had been injected with MMH at concentrations of 7.5 mg/kg and 15 mg/kg. Modifications of this method to analyze for free MMH in urine and blood of human origin are also discussed.

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PREFACE

This study was sponsored by the Aerospace Medical Research Laboratory, Aerospace Medical Division, Wright-Patterson AFB, Oh. The research was performed in accordance with contract no. F33615-78-C-0515, partially supported by the Laboratory Director's Fund. Mr. Paul L. Sherman and Mr. John Pustinger were project leaders for Monsanto Research Corporation. Mrs. Mary K. Hershey was the principal investigator. Marilyn E. George was the Project Engineer for the Aerospace Medical Research Laboratory. Research included in this technical report was initiated on 1 May 1978 and completed on 30 September 1979.

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INTRODUCTION

A considerable quantity of fuels and propellants is handled each year to support the flight requirements of the Air Force. This handling results in a significant potential for human exposure to toxic materials. An understanding of the metabolism of these materials is needed to support toxicity studies and to assist in defining possible detoxification mechanisms. Studies of these toxic materials require precise methods for the identification and measurement of the materials, their metabolites, and conjugates in biological samples. Techniques for identification and measurement must be specific and sensitive because hydrazine and substituted hydrazine compounds are highly reactive and potentially able to react in vitro with many compounds. Important biotransformation reactions, illustrated in Figure 1, include conjugation with carbonyl compounds, acetylation, hydrolysis, and oxidation.

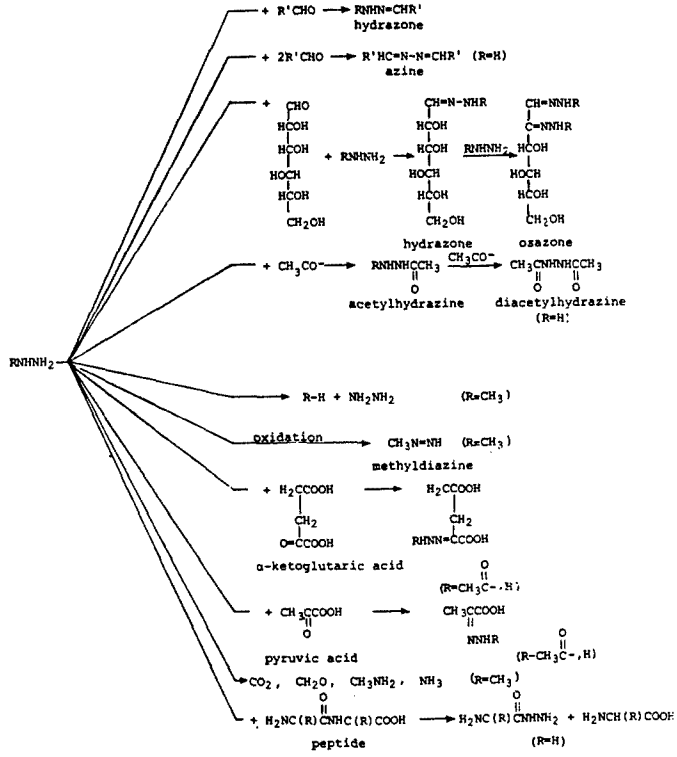


Figure 1. Metabolism of hydrazine compounds.

Reactions with carbonyl compounds produce hydrazones and azine. Reactions with organic acids, acid anhydrides, acid chlorides, esters, and thioesters produce hydrazides. Hydrazines and hydrazides react with natural aldehydes and ketones, including ketonic steroids, to produce hydrazone derivatives. Treatment of deoxyribonucleic acid (DNA) with hydrazine produces apyrimidinic acids (Habermann, 1962) and phenylhydrazine and other substituted hydrazines react with monosaccharides and other carbohydrates to give hydrazones and osazones (Juchau, 1972).

When a peptide is treated with hydrazine, all peptide groups split, and the carbonyl-containing fragment is converted to the corresponding hydrazine (Juchau, 1972). The nonenzymatic reaction of pyridoxal phosphate with hydrazines and hydrazides has also been noted (Juchau, 1972). Hydrazine has been shown to conjugate with acetate and give rise to α-glutamylhydrazide enzymatically (McKennis, 1961; McKennis, 1959).

The C-N bond of hydrazides is labile and susceptible to hydrolysis. The N-N bond is less reactive. The alkyl hydrazine bond is more stable than the C-N hydrazide bond; therefore, monomethylhydrazine (MMH) and 1,1-dimethylhydrazine (UDMH) are more likely to be acetylated than hydrolyzed. However, the formation of methane from MMH has been found to be a significant metabolic pathway (Dost, 1966). Deacetylation of acetylated derivatives is not observed frequently (Juchau, 1972). The autooxidation of MMH yields free radicals capable of DNA interaction. This oxidation can be catalyzed by Cu^{+2}, some metal oxides, and activated carbon (Juchau, 1972).

Hydrazine, in vitro, is acetylated to monoacetylhydrazine, which rapidly converts to diacetylhydrazine. Diacetylhydrazine is excreted unchanged (McKennis, 1959). Due to its reactivity, monoacetylhydrazine cannot be directly determined; however, it has been found excreted in urine as the labile hydrazone of α-ketoglutaric acid and pyruvic acid (Timbrell, 1977). Hydrazine can also form ammonia; it has been reported (Colvin, 1969) that the blood ammonia level of dogs increases upon hydrazine administration. Diacetylhydrazine has no effect on blood ammonia levels.

Monomethylhydrazine forms formaldehyde and methane (Prough, 1970, 1977, 1976, 1969), methylamine (Schwartz, 1966) and carbon dioxide (Dost, 1966). The in vitro reaction of MMH with excess oxygen gave (Saunders, 1976) nitrogen, methane, methanol, ammonia, azomethane, methyldiazine, dimethylamine, formaldehyde methylhydrazone, two isomers of dimethylpiperazine, trimethylpiperazine, trimethylhydrazine, and hydrogen. Methyldiazine ($CH_3N{=}NH$) appeared to be the initial product which was then further oxidized and decomposed. Biological oxidation of alkylhydrazines to hydrocarbons goes by way of alkyldiazines (Tsuji, 1971). Two metabolic routes for MMH have been suggested (Prough, 1973): (1) an alkylhydrazine oxidase converts the alkylhydrazine to the parent alkane, and (2) an N-methylhydrazine demethylase forms formaldehyde from the N-methyl group.

In biological fluids, the most common method of determining metabolized and unchanged hydrazine compounds containing free hydrazine nitrogen is by the analysis of hydrazones after reaction with p-dimethylaminobenzaldehyde (Dambrauskas, 1962, 1964; Reynolds, 1965; Prough, 1977), p-chlorobenzaldehyde (Colvin, 1969), salicylaldehyde (Abdou, 1977), or 2-furaldehyde (Wood, 1976).

The MMH derivative of 2,4,6-trinitrobenzene sulfonic acid has been used for spectrophotometric identification (Prough, 1970), and a qualitative determination of hydrazine, MMH, and UDMH in

the presence of amines has been reported by the color reaction of the hydrazine compound with various aryl nitro compounds (Malone, 1975). Gasometric (McKennis, 1955) and polarographic (Gisclard, 1975) analyses have also been performed.

Timbrell et al. (1977) determined isoniazid, acetylhydrazine, hydrazine, acetylisoniazid, and diacetylhydrazine in human urine using gas chromatography. The first three compounds were analyzed as the p-chlorobenzaldehyde derivative. Diacetylhydrazine and acetylisoniazid were determined in the same manner after acid hydrolysis. An initial methylene chloride extraction removed contaminating substances in the urine.

A linear relationship was found between the peak height of the internal standards (p-bromobenzaldehyde acetylhydrazone and p-bromobenzaldehyde azine) and the derivative. Standard calibration curves were determined showing the ratio of the peak height of the derivative to the peak height of the internal standard versus the concentration of derivative. The sensitivity was 0.4 - 2 µg/ml. Timbrell used a glass column (2m x 1.75 m I.D.) packed with 10% OV-17 on Gas-chrom Q (100 to 120 mesh) and a nitrogen-phosphorus detector.

Dambrauskas and Cornish (1962; 1964) studied the distribution, metabolism, and excretion of hydrazine in rats and mice. Urine was collected in a methanolic solution of p-dimethylaminobenzaldehyde. Immediate derivatization minimized the further reaction of hydrazine after excretion. Analyses of the derivatives were performed spectrophotometrically at 480 nm. Diacetylhydrazine was determined using paper chromatography.

Fiala et al.(1976) separated UDMH metabolites using high pressure liquid chromatography. The separation of UDMH, azomethane, azoxymethane, methylazoxymethanol, methylazoxymethanol acetate, formaldehyde, and methanol was effected on C_{18}/corasil or µBondpak C_{18} columns with 1% ethanol.

Reynolds and Thomas (1965) colorimetrically determined hydrazine and MMH in blood serum. Protein-free serum was prepared by adding 4 ml of 10% aqueous trichloroacetic acid to 1 ml of serum and centrifuging for 6 minutes at 2,000 rpm. An ethanolic solution of p-dimethylaminobenzaldehyde was added to the supernatant, and the derivative was analyzed at 470 nm. The detectable dose level was 3 mg/kg for MMH, and the sensitivity was 0.5 to 10 µg MMH/ml.

In analyzing rat tissues for hydrazine and metabolites, Dambrauskas and Cornish (1964) homogenized the tissue in a p-dimethylaminobenzaldehyde solution. The concentration of the azine derivative was spectrophotometrically determined at 480 nm.

Urine volatiles can be determined (Sucrow, 1973; Shank, 1974) by passing a stream of helium over the top of a mixture of urine and ammonium sulfate and analyzing by GC-MS.

The literature cites relatively few instances of the combination of hydrazine and derivatives with the naturally occuring compounds in the body; however, analysis of these conjugates can be conducted according to one or more of the methods listed in Table 1.

TABLE 1. METABOLITE ANALYSIS OF BIOLOGICAL FLUIDS

Biological fluid analyzed	Method	Ref.	Comment
Urine	Dowex 1-X8	Burtis, 1968	Analyzed constituents of human urine.
Urine	Open tubular glass capillary GC	Horning, 1974a	Analyzed urinary steroids, sugars, metabolites.
Urine, plasma milk		Horning, 1974b	Used salt-solvent pairs to eliminate emulsions and increase extraction yields.
Urine	XAD-2 resin column	Kullberg, 1974	Determine drugs of abuse.
Urine, blood, tissue		Mohan, 1976	Analyzed protein bound metabolites
	GC, HPLC	Still, 1975	Determined pesticide metabolites, particularly polar conjugates and derivatives.
Blood, bile, urine	Sephadex LH-20	Fiala, 1975	Separated polar and non-polar metabolites.
	HPLC: C_{18}/Corosil and μ Bondpak C_{18}	Fiala, 1976	Separation and identification of UDMH metabolites.
Plasma, urine, bile	GC, ion exchange, MS	Bakke, 1976	Review on glucuronide conjugate analysis.

Work was performed in two phases. In the first phase, methods were developed for the analysis of unchanged monomethylhydrazine in the urine and blood from laboratory rats. All rat exposures were conducted by AMRL personnel. The biological fluids were collected and frozen until analyzed. The second phase consisted of modifying the methods developed for the analysis of free MMH in blood and urine of human origin. For these studies, urine samples were provided by Monsanto Research Corporation personnel, and blood serum was furnished by the Community Blood Bank, Dayton, Ohio.

ANALYSIS OF MONOMETHYLHYDRAZINE AND METABOLITES IN RAT URINE

A modification of the method of Timbrell et al. (1977) was selected for analyzing rat urine for MMH and metabolites containing the NH_2 group. The modification consisted of the reaction with p-chlorobenzaldehyde to form hydrazones and p-chlorobenzaldehyde azine, which were recovered by extraction with methylene chloride. Diacetylhydrazine and 1-acetyl-2-methylhydrazine were analyzed as the p-chlorobenzaldehyde derivatives after acid hydrolysis which was effected by acidifying the urine with concentrated HCl and heating at 45°C overnight.

The following derivatives and standards were synthesized:

- p-chlorobenzaldehyde methylhydrazone was prepared (Zlatkis, 1973a) by the reaction of equimolar p-chlorobenzaldehyde and monomethylhydrazine in ether.

- 1-acetyl-2-methylhydrazine was prepared (Condon, 1972) by the reaction of excess ethyl acetate with MMH.

- p-chlorobenzaldehyde 1-acetyl-1-methylhydrazone was prepared by the reaction of equimolar 1-acetyl-1-methylhydrazine and p-chlorobenzaldehyde. The 1-acetyl-1-methylhydrazine was made (Hinman, 1958) by the acetylation of MMH with acetic anhydride in water.

- p-bromobenzaldehyde acetylhydrazone was synthesized (Timbrell, 1977) from equimolar p-bromobenzaldehyde and acetylhydrazine in methanol.

- p-bromobenzaldehyde azine was made (Timbrell, 1977) from stoichiometric amounts of hydrazine hydrate and p-bromobenzaldehyde in methanol.

- p-chlorobenzaldehyde azine was prepared (Timbrell, 1977) from stoichiometric amounts of hydrazine hydrate and p-chlorobenzaldehyde in methanol.

- 1-acetyl-1-methylhydrazine was prepared by the reaction of excess acetic anhydride with MMH.

URINE METABOLITE DERIVATIZATION

Sixteen rats, each weighing ∿225 grams, were injected with MMH, i.p. Eight rats received a dose of 7.5 mg/kg and were placed in metal cages. The remaining eight rats received a dose of 15 mg/kg and were placed in plastic metabolic cages. The control urine was the pooled urine collected from each group during the night prior to MMH injection. Urine samples were collected at two-hour intervals from the time of injection until 16 hours after injection.

Samples were then collected at the intervals of 16 to 24, 24 to 28, 28 to 32½, 32½ to 48, 48 to 56½, and 56½ to 72 hours after injection. It was noted that the urine output from these two groups of rats was much less than expected; the output was particularly low from the 15 mg/kg group. The lowered output could be partially due to the rats infestation with lung mites and internal parasites.

A solution of 25 mg p-chlorobenzaldehyde (PCBA) in 0.1 ml of methanol was added to each milliliter of freshly voided urine. After thorough mixing, the samples were frozen until time for workup. The samples were then thawed, brought to pH 3 with HCl, and reacted for 2.5 hours at room temperature. The metabolite

derivatives were extracted from the urine with methylene chloride, dried (Na_2SO_4), filtered, and rotary evaporated. Prior to gas chromatographic (GC) analysis, the residue was dissolved in 1 ml to 5 ml of ethyl acetate, depending on residue weight.

The aqueous phases from the urine collected up to 16 hours after injection were hydrolyzed by the following method. Concentrated HCl, 15 µl/ml of urine, was added, and the samples were heated overnight in a 45°C bath. The pH was adjusted to 3 with NaOH, and each sample was reacted with 25 mg of PCBA per milliliter of urine for 2.5 hours at room temperature. Methylene chloride extraction and evaporation proceeded in the same manner as that used for nonhydrolyzed samples. The sample derivatization and workup are summarized in Table 2.

TABLE 2. SAMPLE DERIVATIZATION AND WORKUP

Sample Number	Time after injection (hr)	MMH dose (mg/kg)	Urine volume (ml)	Nonhydrolyzed CH_2Cl_2 Extraction volume (ml)	Nonhydrolyzed EtOAc Analysis volume (ml)	Hydrolyzed CH_2Cl_2 Extraction volume (ml)	Hydrolyzed EtOAc Analysis volume (ml)
1	7.5 mg/kg control		42.5[a]	5 x 5	1	3 x 5	1
2	0-2	7.5	12.6	5 x 20	3	3 x 20	2
3	2-4	7.5	5.2	5 x 5	1	3 x 5	1
4	4-6	7.5	3.0	5 x 5	1	3 x 5	1
5	6-8	7.5	7.6	5 x 5	1	3 x 5	2
6	8-10	7.5	6.8	5 x 5	1	3 x 5	2
7	10-12	7.5	5.0	5 x 5	1	3 x 5	1
8	12-14	7.5	5.2	5 x 5	1	3 x 5	1
9	14-16	7.5	5.1	5 x 5	1	3 x 5	1
10	16-24	7.5	30.5	5 x 20	5		
11	24-28	7.5	20.0	5 x 20	3		
12	28-32.5	7.5	14.0	5 x 20	3		
13	32.5-48	7.5	62	5 x 30	-[b]		
14	48-56.5	7.5	40	5 x 30	-[b]		
15	56.5-72	7.5	57.5	5 x 30	-[b]		
16	15 mg/kg control		74.0[a]	5 x 5	1	3 x 5	1
17	0-2	15	13.6	5 x 20	2	3 x 20	3
18	2-4	15	6.4	5 x 5	1	3 x 5	1
19	4-6	15	3.2	5 x 5	1	3 x 5	1
20	6-8	15	1.2	5 x 5	1	3 x 5	1
21	8-10	15	2.8	5 x 5	1	3 x 5	1
22	10-12	15	3.6	5 x 5	1	3 x 5	1
23	12-14	15	5.0	5 x 5	1	3 x 5	2
24	14-16	15	2.8	5 x 5	1	3 x 5	2
25	16-24	15	17.8	5 x 20	3		
26	24-28	15	6.6	5 x 5	1		
27	28-32.5	15	6.9	5 x 5	1		
28	32.5-48	15	25	5 x 20	-[b]		
29	48-56.5	15	12.5	5 x 20	-[b]		
30	56.5-72	15	50.5	5 x 30	-[b]		

[a] 5 ml aliquot used
[b] not analyzed

URINE METABOLITE ANALYSIS

Ethyl acetate solutions of the derivatized urine samples were analyzed using gas chromatography in a differential mode at the following conditions:

Instrument:	Hewlett-Packard 5710A
Column:	6 ft x 2 mm glass, 3% SP-1000 on 100/120 Supelcoport
Carrier:	Helium, 30 ml/min
Injector:	250°C
Detector:	FID, 300°C
Column Program:	100 to 250°C at 8°/min; held at 250°C for 8 min

The results of these analyses are listed in Tables 3 and 4 and illustrated in Figures 2 through 10. Figures 2 through 5 show µg MMH and µg/ml MMH found in nonhydrolyzed and hydrolyzed urine from the 7.5 and 15 mg/kg injected rats. The 7.5 mg/kg injected rats excreted the maximum µg MMH in the interval 2 to 4 hours after injection. The maximum µg MMH output for the 15 mg/kg injected rats occurred 4 to 6 hours after injection. The maximum µg MMH/ml urine occurred in the 2- to 4-hour fraction for the 7.5 mg/kg rats and in the 6- to 8-hour fraction for the 15 mg/kg rats. The result for the 15 mg/kg rats may be somewhat misleading because the combined urine output for these eight animals was only 1.2 ml for this 2-hour interval.

TABLE 3. GAS CHROMATOGRAPHIC ANALYSIS OF NONHYDROLYZED URINE FROM RATS INJECTED WITH MONOMETHYLHYDRAZINE AT THE 7.5 MG/KG LEVEL.

Time after injection (hr)	Urine volume (ml)	MMH[a] (µg)	MMH[a] (µg/ml)	HYD[b] (µg)	HYD[b] (µg/ml)	ACH[c] (µg)	ACH[c] (µg/ml)	AMH[d] (µg)	AMH[d] (µg/ml)
0-2	12.6	151.1	12.0	22.8	1.8	-	-	-	-
2-4	5.2	448.0	86.2	29.2	5.6	9.8	1.9	<0.4	<0.08
4-6	3.0	166.2	55.4	17.9	6.0	4.0	1.3	0.4	0.08
6-8	7.6	147.3	19.4	16.4	2.2	-	-	-	-
8-10	6.8	51.5	7.6	7.9	1.2	-	-	-	-
10-12	5.0	23.6	4.7	3.6	0.7	-	-	-	-
12-14	5.2	7.4	1.5	2.2	0.4	-	-	-	-
14-16	5.1	6.2	1.2	1.7	0.25	-	-	-	-
16-24	30.5	-	-	-	-	-	-	-	-
24-28	20.0	-	-	-	-	-	-	-	-
28-32.5	14.0	-	-	-	-	-	-	-	-

[a]MMH: monomethylhydrazine, detected as PCBA monoethylhydrazone.

[b]HYD: hydrazine, detected as PCBA azine.

[c]ACH: acetylhydrazine, detected as PCBA acetylhydrazone.

[d]AMH: 1-acetyl-1-methylhydrazine, detected as PCBA 1-acetyl-1-methylhydrazone.

TABLE 4. GAS CHROMATOGRAPHIC ANALYSIS OF NONHYDROLYZED URINE FROM RATS INJECTED WITH MONOMETHYLHYDRAZINE AT THE 15 MG/KG LEVEL.

Time after injection (hr)	Urine volume (ml)	MMH[a] (µg)	MMH[a] (µg/ml)	HYD[b] (µg)	HYD[b] (µg/ml)	ACH[c] (µg)	ACH[c] (µg/ml)	AMH[d] (µg)	AMH[d] (µg/ml)
0-2	13.6	204.0	15.0	10.7	0.8	-	-	-	-
2-4	6.4	442.5	69.2	32.1	5.0	15.6	2.4	6.6	1.0
4-6	3.2	514.0	160.6	39.2	12.2	12.7	4.0	10.8	3.4
6-8	1.2	226.0	188.4	9.7	8.1	-	-	0.4	0.3
8-10	2.8	144.5	51.6	16.1	5.8	-	-	-	-
10-12	3.6	111.4	31.0	8.6	2.4	-	-	-	-
12-14	5.0	70.8	14.2	6.5	1.2	-	-	-	-
14-16	2.8	37.1	13.3	6.5	2.3	-	-	-	-
16-24	17.8	25.2	1.4	12.2	1.1	-	-	-	-
24-28	6.6	-	-	1.3	0.2	-	-	-	-
28-32.5	6.9	-	-	0.8	0.1	-	-	-	-

[a]MMH: monomethylhydrazine, detected as PCBA monomethylhydrazone.

[b]HYD: hydrazine, detected as PCBA azine.

[c]ACH: acetylhydrazine, detected as PCBA acetylhydrazone.

[d]AMH: 1-acetyl-1-methylhydrazine, detected as PCBA 1-acetyl-1-methylhydrazone.

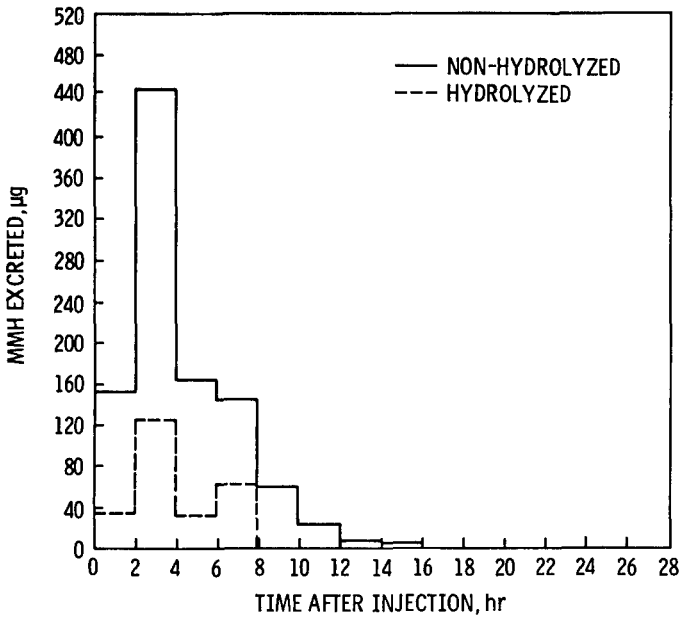


Figure 2. Quantity of monomethylhydrazine excreted in rat urine after monomethylhydrazine injection at 7.5 mg/kg.

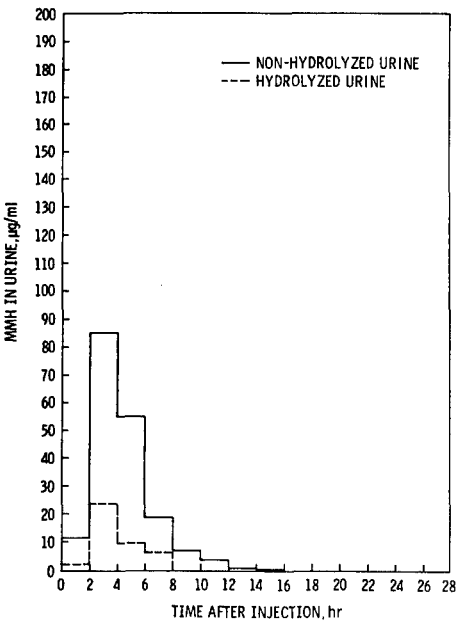


Figure 3. Concentration of monomethylhydrazine in rat urine after monomethylhydrazine injection at 7.5 mg/kg.

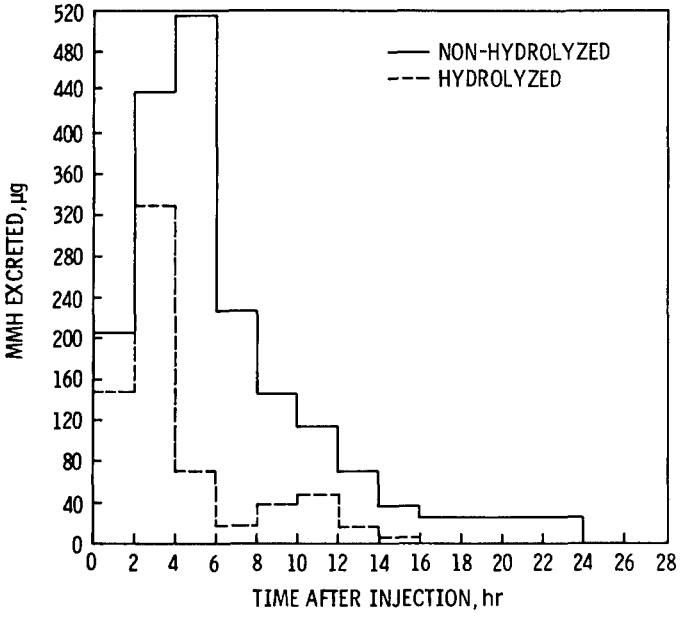


Figure 4. Quantity of monomethylhydrazine excreted in rat urine after monomethylhydrazine injection at 15 mg/kg.

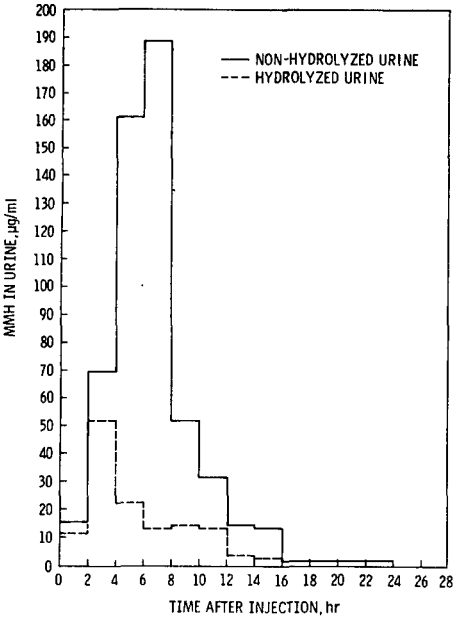


Figure 5. Concentration of monomethylhydrazine in rat urine after monomethylhydrazine injection at 15 mg/kg.

The maximum µg MMH found in the hydrolyzed urine occurred 2 to 4 hours after injection for both groups of rats. This MMH could be from: (1) the hydrolysis of 1-acetyl-2-methylhydrazine, or (2) the hydrolysis of other hydrazones or conjugates that had reacted with MMH. Figure 6 illustrates the total µg MMH from nonhydrolyzed and hydrolyzed urine for both groups of rats.

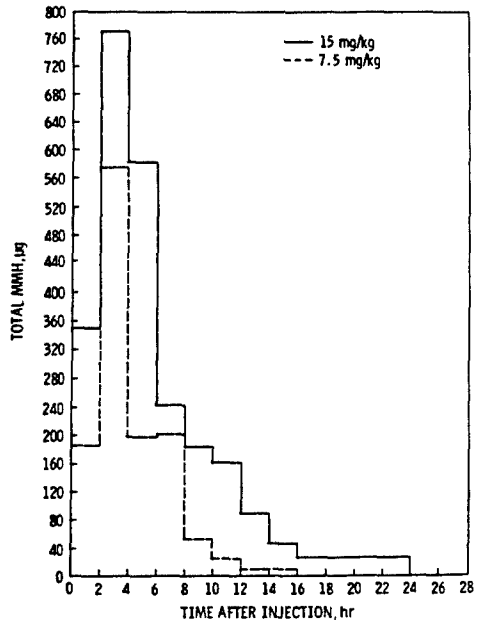


Figure 6. Quantity of monomethylhydrazine in rat urine from hydrolyzed and nonhydrolyzed fractions.

Hydrazine (as the p-chlorobenzaldehyde azine) was found in the nonhydrolyzed urine from both groups of rats. Figure 7 shows that the maximum µg hydrazine was found in the 2- to 4-hour fraction from the 7.5 mg/kg rats and in the 4- to 6-hour fraction from the 15 mg/kg rats. The maximum µg/ml hydrazine (Figure 8) was found in the 4- to 6-hour fractions for both groups of rats. Small amounts of acetylhydrazine and 1-acetyl-1-methylhydrazine were also found as illustrated in Figures 9 and 10.

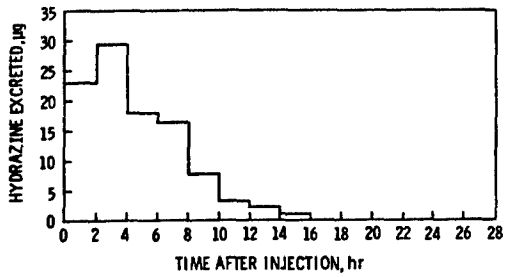


Figure 7a. Quantity of hydrazine excreted in rat urine after monomethylhydrazine injection at 7.5 mg/kg.

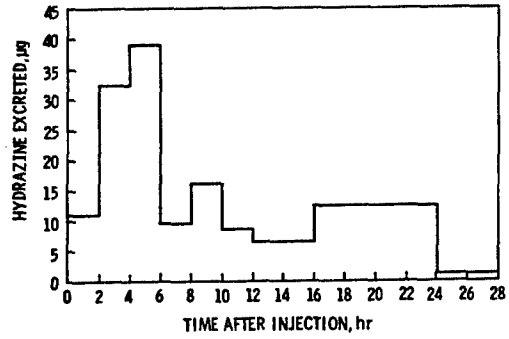
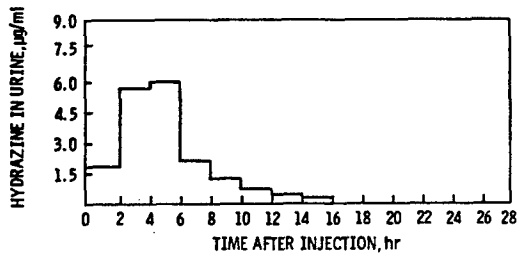
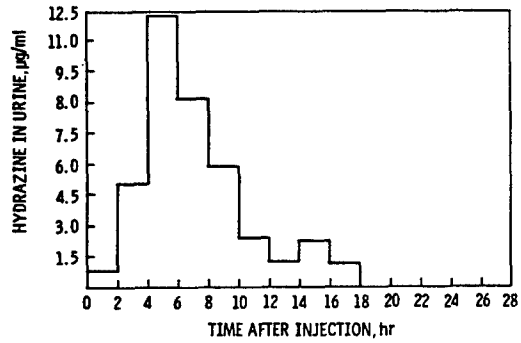


Figure 7b. Quantity of hydrazine excreted in rat urine after monomethylhydrazine injection at 15 mg/kg.

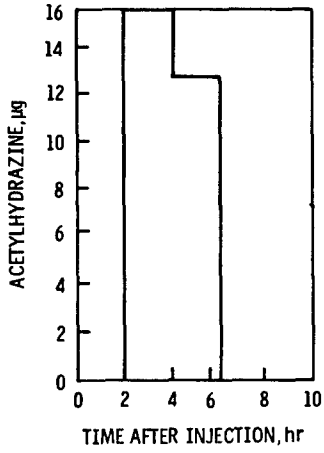


a. 7.5 mg/kg level.

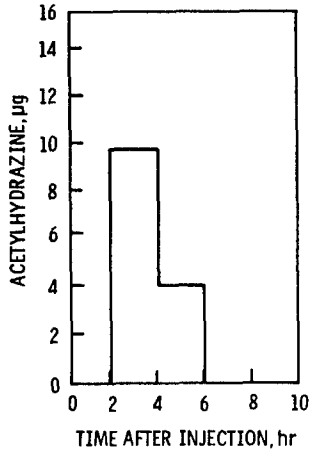


b. 15 mg/kg level.

Figure 8. Concentration of hydrazine found in rat urine after monomethylhydrazine injection.

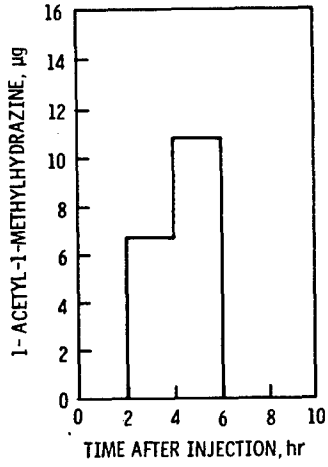


a. 15 mg/kg level.

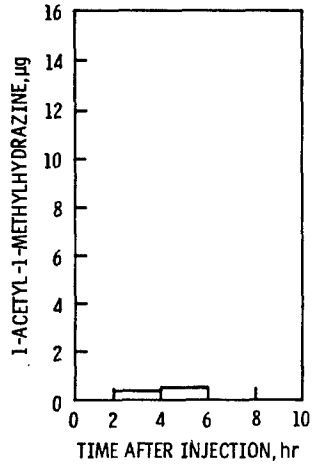


b. 7.5 mg/kg level.

Figure 9. Quantity of acetylhydrazine in rat urine after monomethylhydrazine injection.



a. 15 mg/kg level.



b. 7.5 mg/kg level.

Figure 10. Quantity of 1-acetyl-1-methylhydrazine excreted in rat urine after monomethylhydrazine injection.

BLOOD METABOLITE DERIVATIZATION AND ANALYSIS

Two rats, each weighing 225 grams, were injected with 15 mg/kg MMH, i.p. After one hour, the rats were sacrificed, and the combined blood serum was frozen until analyzed. Blood serum from two control rats was also collected.

The control and MMH-exposed sera were derivatized as follows. Six milliliters of ethanol was added to 2 ml of serum in a culture tube. The precipitated protein material was centrifuged, and the supernatant was adjusted to pH 3 with dilute HCl. p-Chlorobenzaldehyde (25 mg/ml serum) was added and reacted for 2.5 hours at 25°C. The homogenous solution was evaporated and the residue transferred to a culture tube with 1.0 ml of water. This aqueous phase was extracted with four 2-ml portions of methylene chloride. After drying (Na_2SO_4), the combined methylene chloride extract was rotary evaporated in a 0.6-ml capacity vial. This residue was dissolved in 0.4 ml of ethyl acetate for gas chromatographic analysis. Gas chromatographic analysis showed 0.1 µg/ml MMH in the serum.

The aqueous phase was acid hydrolyzed and derivatized. Analysis showed no MMH in this fraction.

VOLATILITY STUDIES ON RAT URINE

Zlatkis and coworkers (McKennis, 1959; Zlatkis, 1971, 1973b, 1973c, 1975, 1976; Stafford, 1976) have studied the analysis of volatile compounds present in biological fluids. The initial technique used was that of head-space analysis in which an inert gas is passed over the top of heated (near 100°C), stirred urine. The volatile compounds, concentrated on a Tenax-GC tube, were analyzed by gas chromatography-mass spectrometry (GC-MS) after thermal desorption.

This initial technique required up to 20 ml of biological fluid per analysis. Modifying this procedure, Andrawes (1977) analyzed 2 ml to 3 ml of urine using a gas-phase stripping technique. The inert carrier gas was bubbled through glass beads, and the sample volatiles were trapped on a Tenax-GC tube. The glass beads served to reduce foam formation.

A modification of the method of Andrawes (1977) was used to concentrate volatiles from a water blank, control urine, and urine from MMH injected rats. Two milliliters of urine and three milliliters of deionized water were placed in a gas-phase stripping (sparging) apparatus (Figure 11) consisting of a tube (1.2 x 1.5 cm) with a 3.5-cm expansion bulb at the top and a coarse glass frit at the bottom. The nitrogen carrier, at a flow of 5 to 20 ml/min depending upon foam production, passed through the glass frit producing small bubbles and enabling faster equilibrium by increasing the surface area of contact between the carrier gas and the urinary components. The volatiles were concentrated on a Tenax-GC tube (Figure 12).

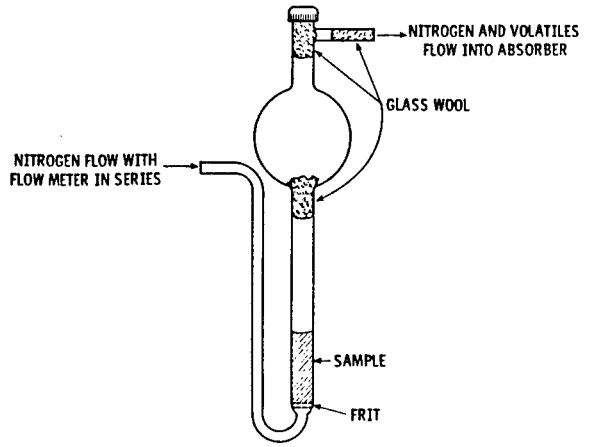


Figure 11. Sparging apparatus for urine volatile analysis.

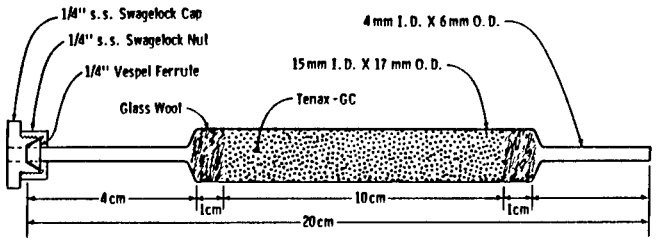


Figure 12. Pyrex sampling tube packed with Tenax-GC.

The tubes were thermally desorbed on a Tenax-GC column (2.1 mm x 1.8 m glass) held at -50°C and connected to a Hewlett-Packard Model 5982 GC-MS. The GC column was programmed from -50°C at 16°C/min to 280°C. The upper temperature hold time was 20 min. Carrier gas was helium at a flow rate of 35 ml/min. Mass spectra were obtained for a scan range of 35 to 380 amu (atomic mass units).

The compounds found in the control urine before injection with MMH, within 0 to 2 hours after injection, and within 4 to 6 hours after injection are shown in Tables 5 through 7, respectively. The compounds found remain essentially constant for the three samples. However, the aromatic compounds were definitely lower in concentration in the samples taken after injection. These data agree with findings during the NH_2-derivative studies. MMH was not found in the urine volatiles.

TABLE 5. VOLATILE COMPOUNDS FOUND IN URINE FROM RATS BEFORE INJECTION WITH MMH

Retention times (min)	Compounds
~12.5	Acetone Methylene chloride (trace)
12.9	Carbon disulfide
~14.7	Methyl ethyl ketone Trace oxygenates of MW 70 and 88
~16.2	Methyl propyl ketone Benzene Trichloroethylene
~18.0	Aliphatic, 57, 43 (~40), 56 (~40), 71 (~35) Toluene Tetrachloroethylene
18.7	Heptanone
~20.9	Unknown, strong 86, 57, 43, 71 ions (resembles types of ketones) C_3-alkyl benzenes
21.4	Weak mixed aliphatics
~21.9	Phenol Benzaldehyde Dichlorobenzene C_4- alkyl benzenes
22.5	Unknown, strong 43, 71 (72), 86 (19) (resembles types of ketones)
23.5	Cresol Acetophenone
24.2	Ethyl phenol
28.0	Unknown[a], strong 71, 43 (42), 41 (8), 243 (5)

[a]Possibly a di-isobutyrate.

TABLE 6. VOLATILE COMPOUNDS FOUND IN URINE FROM RATS 0 TO 2 HOURS AFTER INJECTION WITH MMH

Retention times (min)	Compounds
~13.2	Acetone Methylene chloride (trace)
14.0	Carbon disulfide
15.4	Methyl ethyl ketone
~17.0	Methyl propyl ketone Benzene Trichloroethylene
~19.0	Aliphatic, 57, 45 (40), 96 (40), 71 (35) Toluene Tetrachloroethylene
20.0	Heptanone
~21.8	Unknown, strong 86, 57, 43, 71 ions (resembles types of ketones) C_3-alkyl benzenes
~22.2	Weak mixed aliphatics Weak C_3-alkyl benzenes
~22.9	Benzaldehyde Phenol Dichlorobenzene C_4-alkyl benzenes
~24.0	Cresol Acetophenone
29.6	Unknown[a], strong 71, 43 (37), 56 (7), 159 (7), 243 (4)

[a]Possibly a di-isobutyrate.

TABLE 7. VOLATILE COMPOUNDS FOUND IN URINE FROM RATS 4 TO 6 HOURS AFTER INJECTION WITH MMH

Retention times (min)	Compounds
12.6	Acetone
14.9	Methyl ethyl ketone
~16.5	Methyl propyl ketone Benzene Trichloroethylene (trace)
~18.6	Aliphatic, 57, 43 (40), 96 (40), 71 (35) Toluene Tetrachloroethylene
19.5	Heptanone
21.2	Dimethyl pyrazine
~22.6	Benzaldehyde Phenol Dichlorobenzene (trace)
~24.0	Acetophenone Cresol
~25.4	Ethyl phenol Weak traces of aliphatics and alkyl benzenes
27.3	Naphthalene
~29.5	Unknown[a], strong 71, 43 (40), 56 (7), 159 (7), 243 (5) Methylnaphthalenes (trace)
~32.6	Biphenyl C_2-alkyl-naphthalenes

[a]Possibly a di-isobutyrate

GAS CHROMATOGRAPHY/MASS SPECTROMETRY ANALYSIS ON DERIVATIZED URINE EXTRACTS

In order to better understand the gas chromatograms obtained of the p-chlorobenzaldehyde derivatized urine extracts, a series of GC-MS runs was made. Figure 13 shows the total ion chromatogram and four mass chromatograms for m/e 168, 165, 137, and 276 for a standard solution of p-chlorobenzaldehyde acetylmethylhydrazone (retention time, 18 min), p-chlorobenzaldehyde acetylhydrazone (retention time, 22 min), and p-chlorobenzaldehyde azine (retention time, 25.5 min).

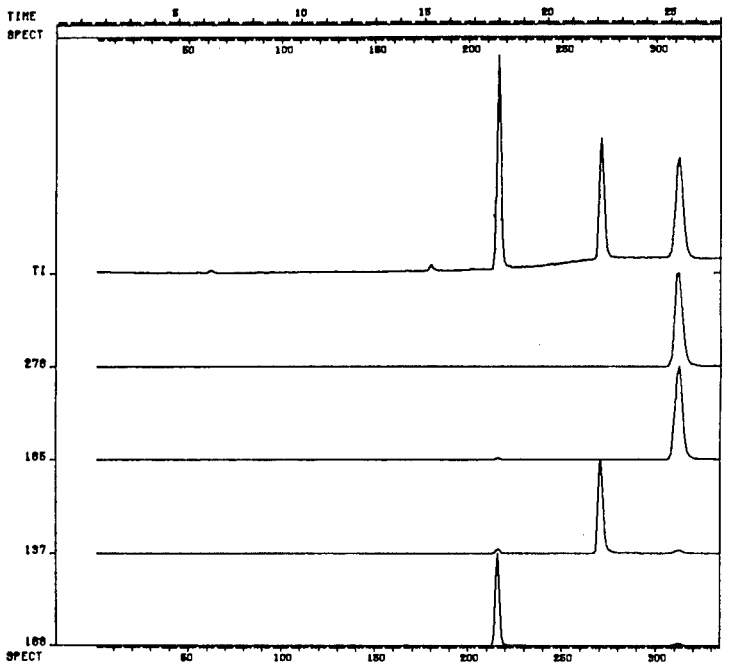


Figure 13. Total ion and mass chromatograms of p-chlorobenzaldehyde standards.

Figure 14, shows the mass spectrum for p-chlorobenzaldehyde acetylmethylhydrazone. An interpretation of the ions found in the spectrum is shown in Table 8. Similarly, in Figures 15 and 16 and Tables 9 and 10, respectively, the mass spectra and interpretations for p-chlorobenzaldehyde acetylhydrazone and p-chlorobenzaldehyde azine are presented.

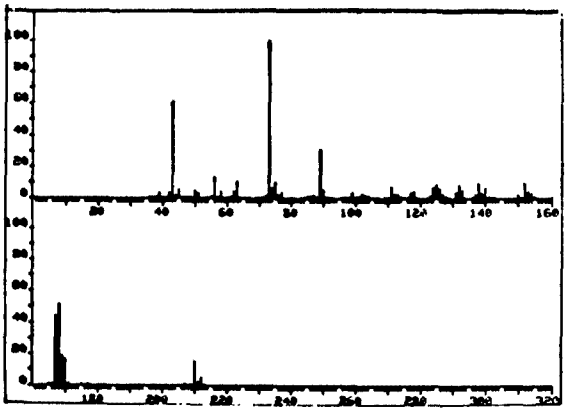


Figure 14. Mass spectrum of p-chlorobenzaldehyde acetylmethylhydrazone

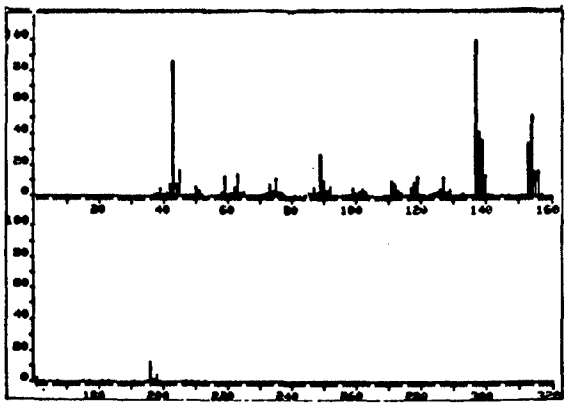


Figure 15. Mass spectrum of p-chlorobenzaldehyde acetylhydrazone.

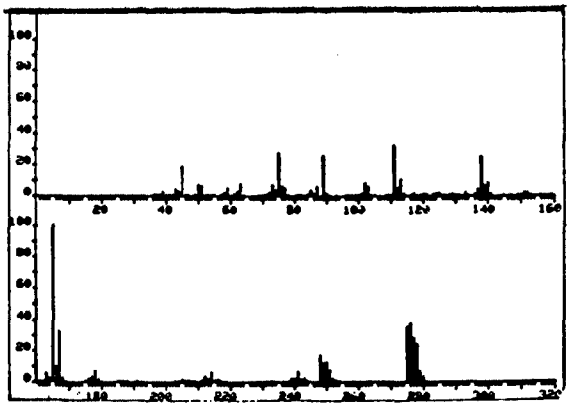


Figure 16. Mass spectrum of p-chlorobenzaldehyde azine

TABLE 8. INTERPRETATION OF MASS SPECTRUM FOR p-CHLORO-BENZALDEHYDE ACETYL-METHYLHYDRAZONE

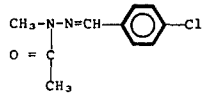
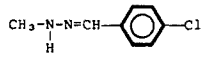
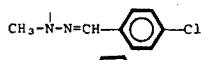
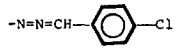
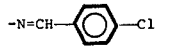
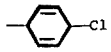
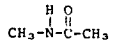
Probable fragment	m/e (chlorine isotope)
	210 (212)
	168 (170)
	167 (169)
	152 (154)
	138 (140)
	111 (113)
	89
	73
	43

TABLE 9. INTERPRETATION OF MASS SPECTRUM FOR p-CHLOROBENZ-ALDEHYDE ACETYLHYDRAZONE

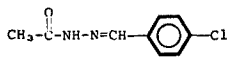
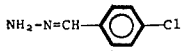
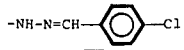
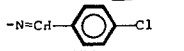
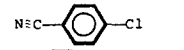
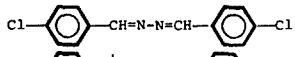
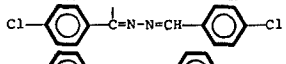
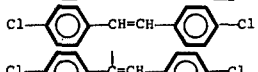
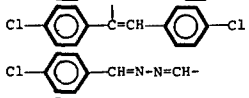
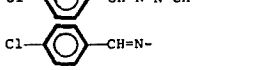
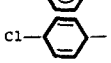
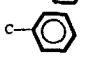
Probable fragment	m/e (chlorine isotope)
	196 (198)
	154 (156)
	153 (155)
	138 (140)
	137 (139)
	89
	43

TABLE 10. INTERPRETATION OF MASS SPECTRUM OF p-CHLORO-BENZALDEHYDE AZINE

Probable fragment	m/e (chlorine isotope)
	276 (278, 280)
	275 (277, 279)
	248 (250, 252)
	247 (249, 251)
	165 (167)
	138 (140)
	111 (113)
	89

One of the p-chlorobenzaldehyde derivatized sample extracts from MMH-injected rats was analyzed by GC-MS. The total ion chromatogram of this sample is shown in the top tracing in Figure 17. The second total ion chromatogram was obtained by subtracting the chromatographic background and expanding (by a factor of eight) the original total ion chromatogram. The four mass chromatograms that make up the balance of Figure 17 are m/e 276, 108, 214, and 165. The rationale for the selection of these mass chromatograms will be explained in the balance of this section. Figure 18 shows the mass spectrum for p-chlorobenzaldehyde (retention time, 6.2 min in Figure 17). The peak at a retention time of 7.0 min is identified as p-chlorophenyl cyanide from its mass spectrum in Figure 19. One of the four peaks between 10 and 12 minutes was identified as p-cresol. The p-cresol retention time was 10.0 minutes, and Figure 20 shows its mass spectrum. The other three peaks in the 10- to 12-minute window were not identified. Figures 21a and 21b show the mass spectra of the peaks at 11.0 and 11.5 minutes.

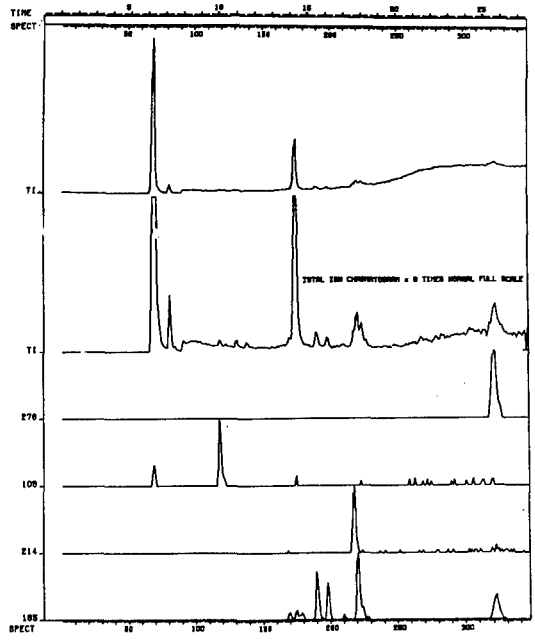


Figure 17. Total ion and mass chromatograms of p-chlorobenzaldehyde derivatized urine extract.

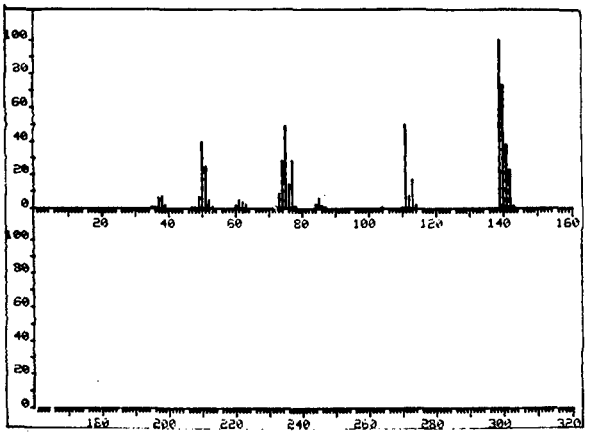


Figure 18. Mass spectrum of p-chlorobenzaldehyde.

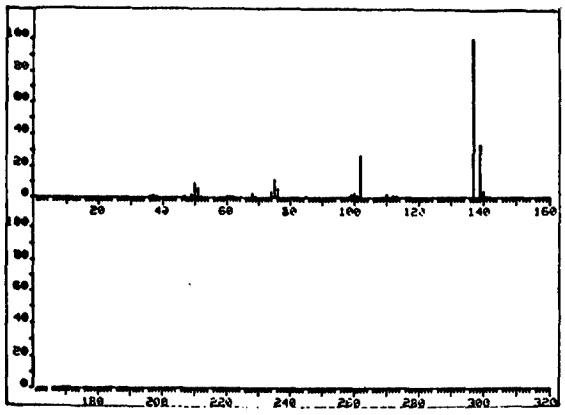


Figure 19. Mass spectrum of p-chlorophenylcyanide.

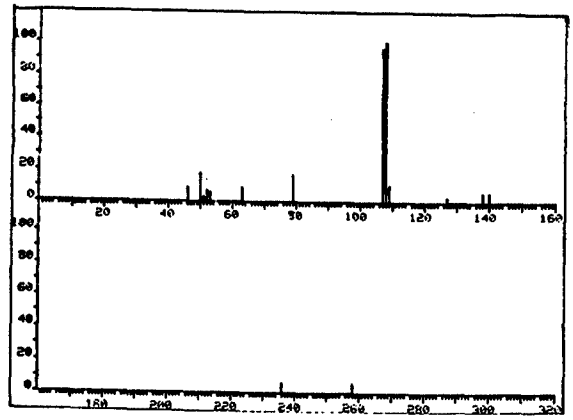


Figure 20. Mass spectrum of p-cresol.

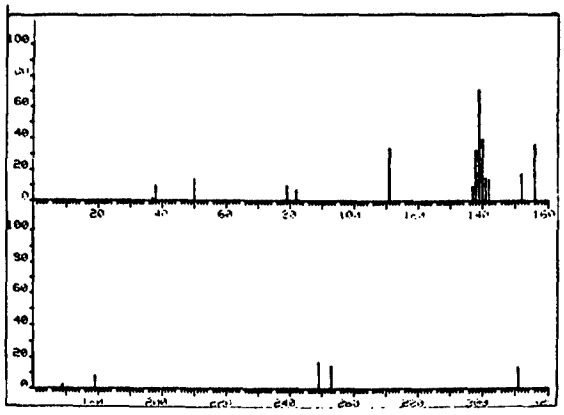


Figure 21a. Mass spectrum of the peak at retention time of 11.0 min in Figure 17.

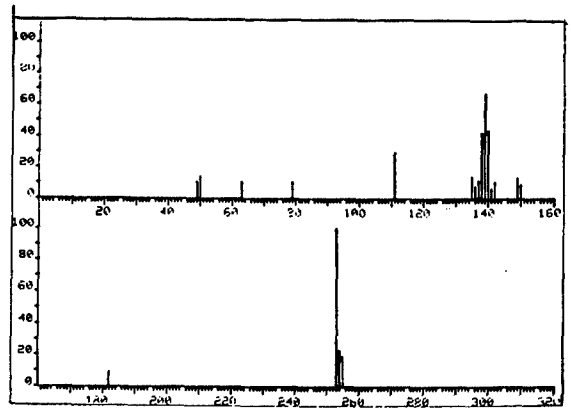


Figure 21b. Mass spectrum of the peak at retention time of 11.5 min in Figure 17.

The mass spectrum of p-chlorobenzaldehyde monomethylhydrazone is shown in Figure 22 and the major m/e's interpreted in Table 11. The peak at ∿17.5 minutes had the mass spectrum shown in Figure 23. Table 12 shows the interpretation of this mass spectrum. The compound was an adduct of aniline and p-chlorobenzaldehyde. Literature shows this type of reaction to proceed exothermally in ∿85% yield. The source of the aniline in the urine is unknown.

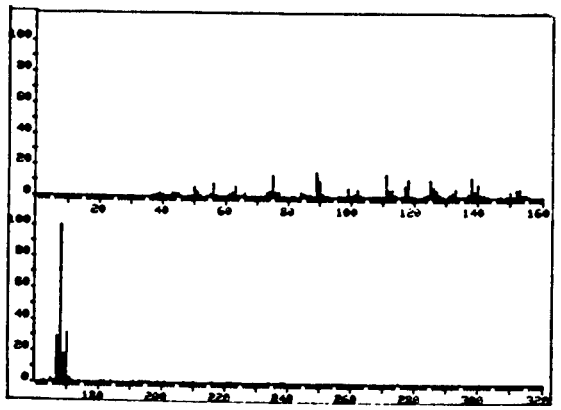


Figure 22. Mass spectrum of p-chlorobenzaldehyde monomethylhydrazone

TABLE 11. MASS SPECTRAL INTERPRETATION FOR p-CHLOROBENZALDEHYDE MONOMETHYLHYDRAZONE

Probable fragment	m/e (chlorine isotope)
CH_3-NH-N=CH-C$_6$H$_4$-Cl	168 (170)
CH_3-N=N-CH-C$_6$H$_4$-Cl	167 (169)
-N=CH-C$_6$H$_4$-Cl	138 (140)
C$_6$H$_4$-Cl	111 (113)
C$_6$H$_5$	89

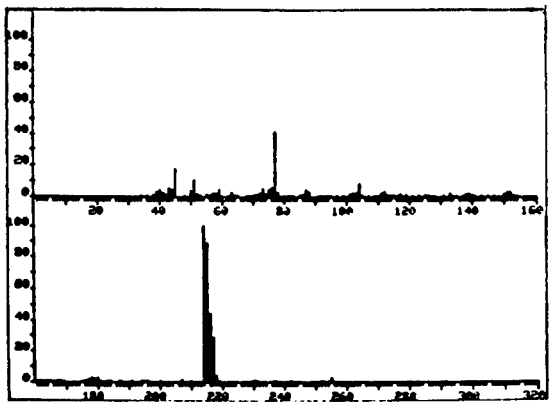


Figure 23. Mass spectrum of peak at 17.5 minutes in Figure 17.

TABLE 12. MASS SPECTRAL INTERPRETATION OF FIGURE 23.

Probable fragment	m/e (chlorine isotopes)
C$_6$H$_5$-N=CH-C$_6$H$_4$-Cl	215 (217)
C$_6$H$_5$-N=C-C$_6$H$_4$-Cl	214 (216)
C$_6$H$_5$-N=CH	104
C$_6$H$_5$	77

The compound C$_6$H$_5$-N=CH-C$_6$H$_5$ shows a very similar mass spectral cracking pattern with the primary ions being due to the molecular weight (M), M-1, m/e=104 and m/e=77.

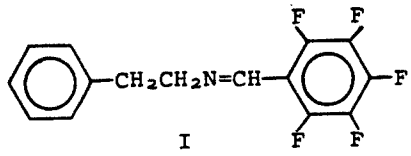
The m/e 214 trace in Figure 17 shows the retention time of the compound just described, m/e 108 illustrates p-cresol, and m/e 276 shows p-chlorobenzaldehyde azine. The remaining mass chromatogram, m/e 165, points out the location of three larger and four smaller peaks. The m/e 165 ion was characterized in Table 10 as the Cl-C$_6$H$_4$-CH=N-N=CH$^+$ ion. A strong definitive mass spectrum was not obtained for any of these seven peaks. Therefore, any interpretation as to the exact identities of these compounds could not be made.

LOW LEVEL MONOMETHYLHYDRAZINE DETERMINATION IN URINE AND BLOOD OF HUMAN ORIGIN

The second phase of this work was concerned with the low-level determination of monomethylhydrazine in human blood and urine.

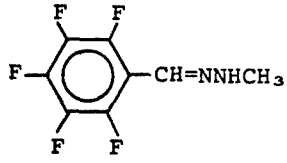
PENTAFLUOROBENZALDEHYDE DERIVATIVE STUDIES

Moffat et al. (1972) reported that pentafluorobenzaldehyde-amine condensation products have good gas chromatographic properties and that their sensitivity of detection by electron capture is approximately 2,000 times that of a flame ionization detector. The types of adducts exhibiting the highest electron capture sensitivity were those having a double bond between nitrogen or oxygen and the carbon atom adjacent to the perfluoro-aromatic ring such as I.



I

For this reason, the pentafluorobenzaldehyde monomethylhydrazone derivative (II) should, likewise, exhibit excellent detection sensitivity with electron capture.



II

A standard solution of pentafluorobenzaldehyde monomethylhydrazone (PFBA-MMH) was prepared according to the method of Moffat (1975) for calibration curve determination and derivative extraction studies. A solution of 25 mg to 50 mg of pentafluorobenzaldehyde (PFBA) and 1.0 mg of monomethylhydrazine (MMH) in 0.2 ml acetonitrile was reacted for one hour at 60°C. Dilutions were made with hexane to obtain a solution of the derivative equivalent to 10 pg MMH/µl. Aliquots of this hexane solution containing 10 pg of 100 pg (equivalent MMH) were analyzed using gas chromatography with electron capture detection at the following conditions:

Instrument:	Hewlett-Packard 5710A
Column:	6 ft x 2 mm glass, 3% SP-1000 on 100/120 Supelcoport
Carrier:	10% methane-90% argon at 37 ml/min
Detector:	Electron capture at 300°C
Column Temperature:	235°C isothermal

The pentafluorobenzaldehyde monomethylhydrazone had a retention time of 7.95 min, and the excess aldehyde had a retention time of 0.30 min under these conditions.

A calibration curve determined by injecting increasing volumes of the derivative standard equivalent to 10 pg to 95 pg MMH had a correlation of 99.96% (r=0.9996).

To determine the reproducibility of derivative extraction from urine, three 2-ml urine samples were spiked with derivative equivalent to 5 mg/ml monomethylhydrazone. Each sample was extracted with 1.0 ml of hexane, and the extract was analyzed in duplicate. The results are shown in Table 13.

TABLE 13. REPRODUCIBILITY OF DERIVATIVE EXTRACTION

			From peak height			
Sample	Theoretical (ng/ml urine)	Found (ng/ml urine)	Mean	S.D.[a]	RSD[b] (%)	Error (%)
1	5	5.7	5.8	0.07	1.2	15
1	5	5.8				
2	5	5.0	5.1	0.07	1.4	1
2	5	5.1				
3	5	5.0	4.9	0.14	2.9	2
3	5	4.8				

[a] S.D. = standard deviation.

[b] RSD = relative standard deviation

A recovery curve was determined by spiking 2-ml urine samples with derivative equivalent to 5 ng/ml, 15 ng/ml, 35 ng/ml, and 45 ng/ml monomethylhydrazone. Hexane extracts were analyzed in duplicate. The recovery curve, showing ng/ml added versus ng/ml found, is shown in Figure 24.

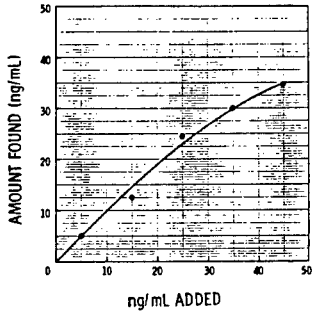


Figure 24. Recovery of pentafluorobenzaldehyde monomethylhydrazone from urine.

In order to determine the recovery of MMH derivatized in urine, four, 2-ml urine samples in a culture tube were charged with 25 ng/ml, 50 ng/ml, 250 ng/ml, and 500 ng/ml MMH. PFBA, 44 µg in 2.5 µl of acetonitrile, was added to each and reacted for one hour at 60°C. Hexane extracts (1-ml) were analyzed for the derivative, PFBA-MMH. The only extract that contained any of the derivative was that from the 500 ng/ml spike, and less than 0.2% of theory was found.

Heating the derivatization mixture at 60°C overnight produced only 37% of the amount of hydrazone that was found after 4 hours at room temperature. A one-hour reaction time at 60°C gave 74% of that formed after 4 hours at room temperature. These results indicated instability in the pentafluorobenzaldehyde monomethylhydrazone that could explain the sometimes irreproducible results obtained when higher temperatures were used.

Further work with lower concentrations of the hydrazone derivative utilized electron capture detection with the gas chromatographic analyses as follows:

Instrument:	Varian 3700
Column:	6 ft x 2 mm glass, 3% OV-101
Carrier:	10% methane - 90% argon at 57 psig
Detector:	Electron Capture at 350°C
Column temperature:	150°C isothermal

Since pentafluorobenzaldehyde was used in large excess, it was necessary to remove that remaining in the hexane extract before analysis of the sample. This was done by treating the hexane extract with a few drops of concentrated aqueous sodium bisulfite solution.

An authentic sample of the pentafluorobenzaldehyde monomethylhydrazone was synthesized using equimolar amounts (0.5 millimole) of pentafluorobenzaldehyde and monomethylhydrazine in 1 ml of methanol. The pure hydrazone precipitated from solution as nearly colorless needles with a melting point of 147 to 148°C. Analytical results are shown in Table 14.

TABLE 14. ELEMENTAL ANALYSIS FOR $C_8H_5N_2F_5$

	Percent			
Data Source	Carbon	Hydrogen	Nitrogen	Fluorine
Theoretical	42.87	2.25	12.50	42.32
Actual[a]	42.81	2.26	12.53	42.58

[a]Analysis performed by Galbraith Laboratories, Inc., Knoxville, TN 37921.

The infrared spectrum of the prepared pentafluorobenzenzaldehyde monomethylhydrazone showed a strong C=N bond at 1585 cm^{-1}, which was consistent with the assigned structure.

Replicate injections of a dilute (0.1 pg/µl) hexane solution of the prepared derivative indicated marked instability of this compound when stored at room temperature. A 0.2-pg injection of a 24-hour old solution gave a GC peak height 38% as large as that obtained with the fresh solution. Storage of the solution at -10°C gave an 11% peak height reduction in 24 hours. The hydrazone appeared more unstable at higher hexane concentrations; a 1 ng/µl solution decomposed within a few hours to only 0.3% of the original value.

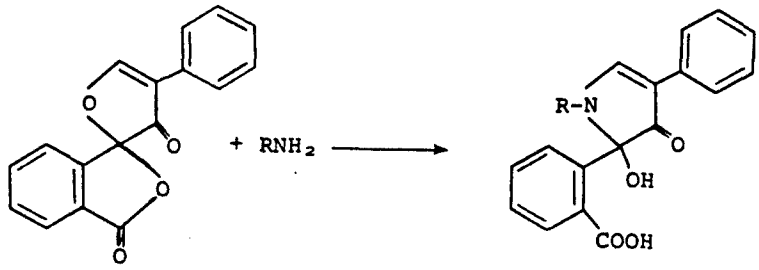
Low-level detection of the pentafluorobenzaldehyde monomethylhydrazone was further complicated by the presence of a naturally occurring component in human urine that combined with the pentafluorobenzaldehyde to form an adduct having the same retention time (2.2 min) as the MMH derivative. The analysis of a hexane extract of a urine blank showed no material eluting with this retention time. Other interferences arise from perfluorinated, highly electron capturing impurities present in the pentafluorobenzaldehyde that are not removed by treatment with sodium bisulfite. Because of the problems encountered with pentafluorobenzaldehyde monomethylhydrazone, further work with this labile derivative was not performed.

The analysis of the p-chlorobenzaldehyde derivative with flame ionization detection appears to be the most reliable gas-chromatographic method of determining monomethylhydrazine in urine. A sensitivity of 1 µg MMH/ml urine can be attained when analyzing 10 to 25 ml urine using the method described in the appendix.

HIGH PRESSURE LIQUID CHROMATOGRAPHIC TECHNIQUES

Fluorescamine Derivative Studies

Fluorescamine, 4-phenylspiro[furan-2(3H),1'-phthalanl]-3,3'-dione, has been used as a reagent for the assay of amino acids, peptides, proteins, and primary amines in the picomole range (Udenfriend, 1972; DeBernardo, 1974; Stein, 1974; Haefelfinger, 1975; Imai, 1975; Schmidt, 1974). Fluorescamine and its hydrolysis products are nonfluorescent while the primary amine adduct is strongly fluorescent (390 nm excitation, 475 nm emission). This reaction is said to occur in a fraction of a second at room temperature (Udenfriend, 1972).



FLUORESCAMINE

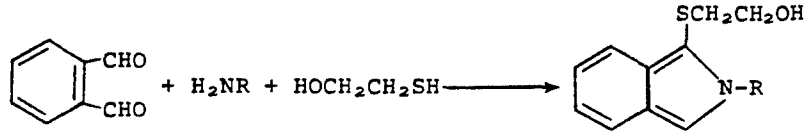
To test the applicability of this method to low-level MMH detection, the fluorescamine-MMH derivative was prepared by reacting 0.11 mg fluorescamine and 4.4 µg MMH in 1.0 ml of acetonitrile. Analysis of this acetonitrile solution by high pressure liquid chromatography (HPLC) was made at the following conditions:

Column:	LiChrosorb RP-18
Solvent:	70% acetonitrile
Flow:	1 ml/min
UV detection:	254 nm; range: 0.02 aufs
Fluorescent detection:	286 nm; Schoeffel no. 360 filter; range: 0.05 µA

Under these conditions, an injection equivalent to 22 ng of MMH gave a 9-cm peak with the fluorescent detector and a 7.3-cm peak with UV.

o-Phthalaldehyde Derivative Studies

Picomole quantities of amines, amino acids, and proteins have been detected by reaction with o-phthalaldehyde and thiols (Davis, 1979; Simons, 1976; Roth, 1971; Weeks, 1976). Davis et al. (1979) determined biogenic amines in plasma, tissue, and urine by precolumn derivatization with o-phthalaldehyde and 2-mercaptoethanol forming a strong fluorescent adduct (III).



III

Since fluorescent spot testing of hydrazines with fluorescamine and isomeric phthalaldehydes has been reported (Weeks, 1976), it appeared that this technique would be applicable to the low-level detection of MMH. However, preparation of the o-phthalaldehyde derivative by the method of Davis (1979) and analysis by HPLC gave inconclusive results as to the applicability of this method for low-level MMH detection.

CONCLUSION

Subpicogram levels of synthesized pentafluorobenzaldehyde monomethylhydrazone have been detected by gas chromatographic analysis with electron capture detection. The limiting factors in the in situ derivatization of MMH in urine with pentafluorobenzaldehyde are derivative instability, the impurities found in the aldehyde, and the formation of aldehyde complexes with compounds normally found in human urine which elute from the GC column at the same time as the MMH derivative.

The p-chlorobenzaldehyde monomethylhydrazone has been shown to be a relatively stable MMH derivative. A sensitivity of 1 μg MMH/ml urine can be attained when analyzing 10 to 25 ml of urine with flame ionization detection. Although the sensitivity of this derivative to electron capture detection would be less than the pentafluorobenzaldehyde derivative, the increased derivative stability makes for a more reliable MMH detection method. Similarly, the p-dimethylaminobenzaldehyde monomethylhydrazone could be prepared and analyzed with a nitrogen specific detector on the gas chromatograph.

Optimization of the fluorescamine monomethylhydrazine derivative formation and subsequent HPLC analysis could conceivably give sensitivities in the ng/ml range. Since the sensitivity of fluorescent detection is in the ng level, derivatization of larger volumes of urine followed by appropriate extraction and derivative concentration procedures would be necessary.

APPENDIX

METHOD FOR THE ANALYSIS OF MONOMETHYLHYDRAZINE (MMH) IN URINE (AND BLOOD)

METHOD

SCOPE

This method was developed for the analysis of monomethylhydrazine in human urine and is applicable for the detection of monomethylhydrazine in urine at concentrations of 1 mg/ml to 1 µg/ml. A urine volume of 1.0 ml is sufficient for higher MMH concentrations. For lower concentrations, 10 to 25 ml urine should be analyzed.

The method can be adapted for the detection of monomethylhydrazine in serum.

SUMMARY OF METHOD

The monomethylhydrazine is derivatized in urine with p-chlorobenzaldehyde. The derivative, p-chlorobenzaldehyde monomethylhydrazone, is removed by methylene chloride extraction. The derivative is measured by gas chromatography with flame ionization detection.

INTERFERENCES

Freedom from interference should be demonstrated by the analysis of urine blanks. The urine extracts should be kept frozen and the analysis performed as soon as possible after extraction.

APPARATUS

Screw cap culture tubes (16 x 100 mm) with Teflon-lined caps.

Centrifuge

Gas chromatograph equipped with flame ionization detector.

Glass column for gas chromatograph (6 ft x 2 mm) packed with suitable packing.

REAGENTS AND MATERIALS

GC column packing, 3% SP-1000, 100/120 mesh on Supelcoport, Supelco, Inc.

p-Chlorobenzaldehyde, Matheson.

Methylene chloride, Burdick and Jackson "Distilled-in-Glass" or equivalent.

Monomethylhydrazine, Eastman.

Acetonitrile, Burdick and Jackson "Distilled-in-Glass" or equivalent.

Stock standard solution: Prepare a stock standard solution (1 mg/ml) of the derivative by reacting 25 mg p-chlorobenzaldehyde and 1 mg monomethylhydrazine in 1 ml acetonitrile for 2.5 hour. The extract is stored in a freezer until used.

Working standards: Pipette 10 µl of the 1 mg/ml standard solution into 1.0 ml acetonitrile for a concentration of 10 ng/µl. Other concentrations may be prepared, if more appropriate.

CALIBRATION

Calibration Curve: The linearity of response of the detector is determined by injecting various amounts of the working standard into the chromatograph. The peak height of the derivative is measured.

Linearity can be established by plotting a calibration curve of the amount of derivative injected versus the peak height measured. Samples which have peak heights outside the linear range should be diluted by an appropriate factor and reinjected.

PROCEDURE

Derivatization of monomethylhydrazine

p-Chlorobenzaldehyde, 25 mg, is placed in a culture tube purged with nitrogen.

Add urine (1.0 to 25 ml) to the culture tube and bring to pH 3 with dilute HCl. Shake vigorously for 1 to 2 minutes, and let stand at room temperature for 2.5 hours with intermittent shaking.

Extraction of derivative

Add methylene chloride (5.0 ml) to the culture tube, shake vigorously for 2 minutes, and centrifuge the sample to break up any emulsion which might have formed. Remove the organic layer and repeat the extraction 4 times. Evaporate the extract on a rotary evaporator and dissolve the residue in 1 ml ethyl acetate. Freeze until ready for analysis.

CHROMATOGRAPHY OF EXTRACTS

The following conditions have been found suitable for this method.

Column:	6 ft x 2 mm glass column packed with 3% SP-1000 on 100/120 mesh Supelcoport
Carrier Gas:	Helium, 30 ml/min
Injection Port Temp:	250°C
Detection Temp:	250°C
Column Program:	100°C to 250°C at 8°/min; held at 250°C for 8 min.

Inject a suitable aliquot (e.g., 2 µl) of the sample into the gas chromatograph, and measure the heights of the peaks eluting at the retention time of the derivative. If the peak height falls outside the linear range, the sample should be diluted and reinjected. Calibration curve should be prepared daily with freshly prepared Standard Stock solution.

CALCULATION OF RESULTS

The quantity of monomethylhydrazine in the injected sample can be determined by direct comparison with the calibration curve. The concentration of monomethylhydrazine present in the urine sample can be determined by the following equation:

$$C_u(\mu g/ml) = \frac{W_I \times V_H \times DF}{V_I \times V_u}$$

where
- C_u = monomethylhydrazine concentration present in the urine.
- W_I = quantity (ng) in the injected sample.
- V_H = volume (ml) of ethyl acetate solution.
- DF = dilution factor.
- V_I = injection volume (µl).
- V_u = volume (ml) of urine analyzed.

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